

## Video Article

# Measuring Stolons and Rhizomes of Turfgrasses Using a Digital Image Analysis System

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## Abstract

Length and diameter of stolons or rhizomes are usually measured using simple rulers and calipers. This procedure is slow and laborious, so it is often used on a limited number of stolons or rhizomes. For this reason, these traits are limited in their use for morphological characterization of plants. The use of digital image analysis software technology may overcome measurement errors due to human mistakes, which tend to increase as the number and size of samples also increase. The protocol can be used for any kind of crop but is particularly suitable for forage or grasses, where plants are small and numerous. Turf samples consist of aboveground biomass and an upper soil layer to the depth of maximum rhizome development, depending on the species of interest. In studies, samples are washed from the soil, and stolons/rhizomes are cleaned by hand before analysis by digital image analysis software. The samples are further dried in a laboratory heating oven to measure dry weight; therefore, for each sample, the resultant data are total length, total dry weight, and average diameter. Scanned images can be corrected before analysis by excluding visible extraneous parts, such as remaining roots or leaves not removed with the cleaning process. Indeed, these fragments normally have much smaller diameters than stolons or rhizomes, so they can be easily excluded from analysis by fixing the minimum diameter below which objects are not considered. Stolon or rhizome density per unit area can then be calculated based on sample size. The advantage of this method is quick and efficient measurement of the length and average diameter of large sample numbers of stolons or rhizomes.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/58042/>

## Introduction

The study of plant morphology is largely addressed in all disciplines of plants science including ecology, agronomy, biology, and physiology. The plant root system is widely studied for its importance in stress tolerance, soil stability, plant growth, and productivity. Stolons and rhizomes are also widely studied for their role in plant propagation strategies, recuperative ability, and carbohydrate storage. Stolons and rhizomes are modified stems that grow horizontally, either above-ground (stolons) or below-ground (rhizomes). Stolons and rhizomes also contain regularly-spaced nodes and internodes, as well as meristematic nodes that are capable of giving rise to new roots and shoots<sup>1</sup>. There have been a wide number of studies on different topics investigating roots, stolons, and rhizomes of various plants<sup>2,3,4,5,6,7,8</sup>. Root systems, stolons, and rhizomes of turfgrasses are studied because of their importance in turf quality<sup>9</sup>, spring green-up after winter dormancy<sup>10</sup>, and wear tolerance and recuperative ability<sup>11</sup>. Furthermore, these organs are also studied in other crops, turfgrasses such as rice<sup>12</sup>, soybean<sup>4</sup>, and maize<sup>13</sup>, and pastures where lateral stems play a key role in soil erosion control<sup>5</sup>.

Root length density (root length per soil volume) and average diameter are commonly measured using scanning software<sup>3,4,5,9,14,15,16,17,18</sup>. Conversely, the length and diameter of stolons or rhizomes are usually measured with a ruler and caliper<sup>3,19,20</sup> and require significant time and labor<sup>21,22,23,24</sup>. Hence, they are often measured in a restricted number of stolons or rhizomes<sup>11,20,25</sup> and are often limited to the morphological characterization of spaced plants only. The study of stolon and rhizome traits in a mature canopy involves sampling a large amount of biomass so that usually only stolon and rhizome dry weight density (dry weight per unit of surface) are determined<sup>7,11,26,27</sup>. Stolon dry mass, in fact, can be more easily measured than stolon length and diameter by drying samples in an oven. However, stolon length is an important species and varietal character that is not well-related to dry mass. A recent study on creeping perennial ryegrass (*Lolium perenne*) demonstrated that samples with high stolon length density did not necessarily have high stolon weight density<sup>6</sup>.

Image-analysis systems make the analysis of roots faster<sup>28,29</sup>, more accurate, and less prone to human error<sup>30,21</sup> than traditional, manual methods<sup>31,32,33</sup>. Moreover, these systems provide high flexibility and easy-to-use tools including the light, optical setup, and resolution, which are often calibrated for each scanned image<sup>34</sup>. Pornaro *et al.*<sup>24</sup> demonstrated that the WinRHIZO system, an image analysis system specifically designed for measurement of washed roots, may provide an alternative method to analyze stolon and rhizome traits more completely than current methods by overcoming measurement errors caused by human mistakes. For a morphological description and quantitative information on stolon and rhizome growth, image-analysis systems can be used to analyze a large number of samples quickly, even with a large amount of

biomass, allowing increased statistical precision. Therefore, root analysis software packages provide an alternative, reliable, and fast method for studying the growth and morphology of stolons and rhizomes of different plant species<sup>24</sup>.

We present an experiment carried out in northeastern Italy to study stolon and rhizome development of four cultivars of bermudagrass (*Cynodon* spp.). The study aimed to increase knowledge on the development of stolons and rhizomes in seeded ("LaPaloma" and "Yukon") and vegetative ("Patriot" and "Tifway") cultivars of bermudagrass. The experiment was established in May 2013, and turf samples were collected over three sampling dates per year, from autumn of 2013 to summer of 2015 [March (before the green up), July (full growing season), and October (before winter dormancy)]. For the description and explanation of this method, we used samples collected in the summer of the second growing season (July 2014), as the large biomass of the samples at this time justified the need for a rapid analysis. The WinRHIZO, a digital image-analysis software tool specifically designed for washed root measurements, was used to determine stolon length density and average diameter.

## Protocol

### 1. Collection of Biomass Samples

1. Collect samples including the aboveground biomass and a soil layer with an appropriate depth depending on species (for turf species, a 15 cm depth is generally sufficient) to ensure collection of both stolons and rhizomes.  
Note: Overall plot size must be considered prior to initiating the study, since destructive samples will be taken. In general, the longer the experiment is conducted, the larger the required plot size.
2. Check soil conditions before sample collection: if the soil is too dry, especially in heavy soils, it may be difficult to collect samples. In this case, irrigate the plots before collection to soften sample layers.
3. Collect samples using a soil core sampler ( $\geq 8$  cm diameter) or define the surface area to collect with a frame ( $\geq 10 \times 10$  cm), and collect the samples with a spade. Label each sample with laboratory tape.
4. Collect several random samples per plot so they are representative of the plant population.
5. Use the same sampler for the entire experiment and record the area that each sample represents to calculate stolon and rhizome density.  
Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than  $-18$  °C.

### 2. Cleaning the Biomass Samples

1. Place the sample in a large sieve with 0.5-1.5 mm openings depending on stolon or rhizome size. The openings should be small enough to retain all stolons and rhizomes, but large enough to allow soil particles to be removed. For sandy soils, two sieves with different openings, placed one on top of the other, may allow for better precision and efficiency.
2. Clean the samples with a stream of water with enough power to remove soil particles without damaging the plants.
3. Retrieve the cleaned samples and place them in a tray with paper towels, taking care to label the trays appropriately.  
Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than  $-18$  °C.
4. Further clean the samples by removing roots and leaves with scissors. During this process, separate stolons and rhizomes, if needed, and record additional information such as the numbers of plants, tillers, and stolons per plant.  
Note: Removing all root and leaf tissue from stolons and rhizomes will improve precision. Fine roots are difficult to remove; however, through the digital image analysis, it is possible to omit them from the analysis using a software application that excludes organs with a diameter less than a chosen value (see step 5.1), which is defined fairly accurately based on observations of images reproduced onscreen.
5. Place stolons and rhizomes in paper labeled bags.  
Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than  $-18$  °C.

### 3. Scanning and Image Analysis of Samples

1. Place the sample on a transparent plastic tray of the WinRHIZO standard scanning equipment. Manually place the stolons and rhizomes using laboratory forceps to minimize overlapping. Large samples may need to be split into subsamples.
2. Do not add water to the tray (as recommended for roots), because stolons and rhizomes have sufficient rigidity to avoid excessive proximity of organs which may cause reading errors, which usually happens with fine roots.
3. Place the tray on the scanner surface.
4. Turn on the scanner and start running the program.
5. Check image dpi in the **Image** menu, command **Image acquisition parameter**, for a possible further control in the saved image.
6. Check threshold in **Analysis**, command **Root & Background Distinction**, for good classification of pixel belonging to the scanned organs.
7. Check that the whole tray surface will be scanned in the **Image** menu, command **Image acquisition parameter**.
8. Check diameter class displayed for organs distribution per diameter, in the graphic area above the scanned image. Select 20 equal-width classes with 0.1 mm intervals by clicking on the horizontal axis of the graph. This function allows exclusion of data belonging to roots or small organs, when stolons or rhizomes were not perfectly cleaned. The literature reports that most roots of turf species have diameters lower than 0.2 mm.  
Note: Width and number of classes can be modified while taking into account the average diameter of stolons and rhizomes for the analyzed samples and variability around this mean. A control should be conducted in some samples to determine the minimum diameter to be excluded.
9. Run the first sample scanning and check that the edit allows for a good analysis.
10. Follow the software instructions for saving the image and processed analysis. Label the image and analysis with the sample label.
11. Proceed with the scanning of all samples.  
Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than  $-18$  °C.

## 4. Measurement of Dry Weight

1. Using a precise electronic balance, place the scanned samples in a tared aluminum tray.
2. Repeat step 4.1 for all scanned samples.
3. Insert all the samples into an oven set to 105 °C and dry them for 24 h.
4. Remove the samples and wait until the tissue weight has stabilized.
5. Weigh all the samples with their tare.
6. Subtract the tare from the recorded weight to obtain the net weight of each sample.

## 5. Correction of Data and Calculation of Length and Weight Density

1. Correction of length and averaged diameter
  1. Convert the .txt file resulting from the analysis with WinRHIZO to a .csv file.
  2. Use the results grouped for diameter classes to exclude data of organs smaller than 0.2 mm (roots, part of leaves, or scratches on the tray).
  3. For each WinRHIZO reading (rows of .txt file) sum all the lengths recorded for diameter classes greater than 0.2 mm. The length calculated with this correction is the effective length to be used for further data processing.
  4. For each WinRHIZO reading, sum the projection areas recorded for diameter classes more than 0.2 mm. The proportion between length and projection area gives the average diameter corrected for exclusion of organs with diameters less than 0.2 mm.
2. If the sample has been split into subsamples, calculate the final length as the sum of all subsample lengths, and calculate the final average diameter as the proportion between the sum of all subsample lengths and sum of all subsample projection areas.
3. When necessary, calculate the length and weight density per unit surface area based on sample size.
4. Use the data obtained for statistical analysis.

## Representative Results

A field experiment was established in autumn 2013 to compare stolon and rhizome development of four bermudagrass cultivars, including two seeded types ("LaPaloma" and "Yukon") and two sterile vegetative hybrids ("Patriot" and "Tifway"). The experimental design was a randomized complete block with three replications, for a total of 12 plots (2 x 2 m).

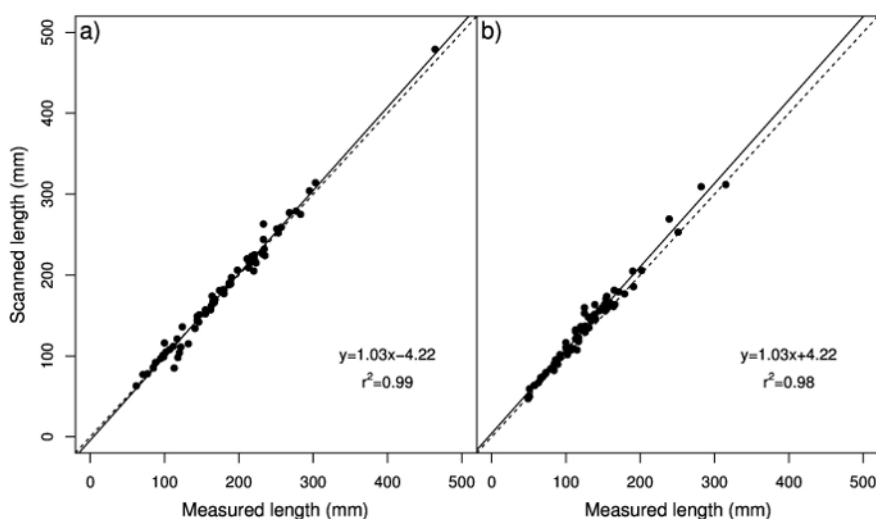
Fourteen stolons and fourteen rhizomes from each turf-type cultivar and the wild bermudagrass were collected randomly in the plots, as well as from wild bermudagrass plants growing near the plots, for a total of 70 stolons and 70 rhizomes. All stolons and rhizomes were cleaned as described in the protocol (step 2) before further measurement. Internode diameter and length were measured with a caliper and ruler, respectively, and the number of nodes was counted for each stolon or rhizome. The times necessary to clean and measure stolon and rhizome samples with the ruler and caliper were also recorded. Stolon and rhizome diameters were calculated as the means of all internode diameters measured. Total stolon and total rhizome lengths were calculated as the sum of all internode lengths. Furthermore, total scanned lengths and scanned diameters of each stolon and rhizome were measured using a digital image analysis system, as described in steps 3 and 5. The times necessary to measure stolon and rhizome traits by the digital analysis system were recorded. Each stolon and rhizome were then cut with scissors to separate internodes from nodes, and the internodes were used to estimate the scanned internode diameter as described in steps 3 and 5. Pearson's correlation coefficients were calculated for stolons and rhizomes ( $n = 70$  stolons,  $n = 70$  rhizomes) between measured and scanned lengths, measured and scanned diameters, number of nodes and the absolute value of the difference between measured and scanned diameters, and measured diameters and scanned internode diameters. The lengths measured with the ruler were used to calibrate the lengths estimated through the digital image analysis system.

The regression analysis indicated a high correlation between stolon scanned length and measured length (**Figure 1a**), with a slope of 1.03 and intercept of -4.22, as well as between rhizome scanned length and measured length (**Figure 1b**), with a slope of 1.03 and intercept of 4.22. Cleaning by hand, 14 stolons and 14 rhizomes took an average time of 21 min and 24 s and 11 min and 12 s, respectively. The average time to measure length and diameter with a ruler and caliper was 14 min and 6 s for stolons and 13 min and 35 s for rhizomes. The scanning and software analysis of samples using WinRHIZO took an average of 11 min for stolons and 12 min and 4 s for rhizomes.

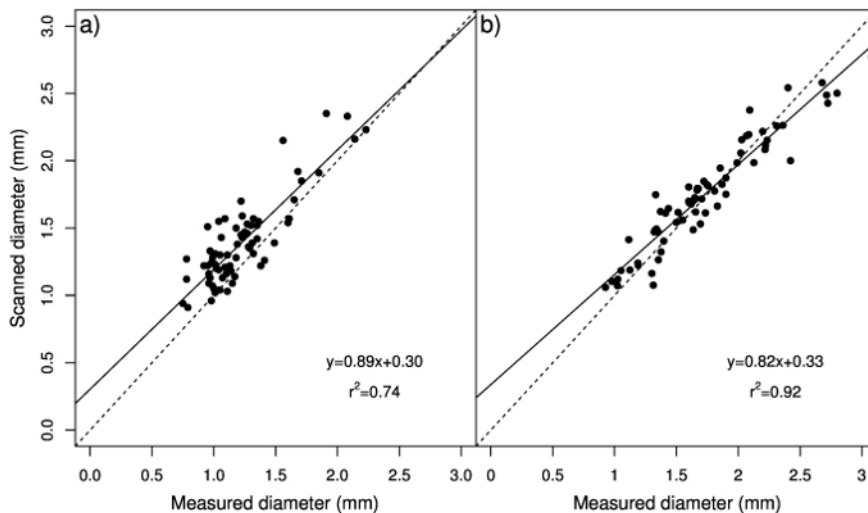
Measured and scanned diameters were also significantly correlated in both stolons and rhizomes. The relationships between measured and scanned diameter were close to 1:1, indicating a good fit of the data (**Figure 2a** and **2b**). However, the intercept indicated that digital image analysis system overestimated measured diameter, especially for lower values, and that higher values of rhizome diameter were underestimated. This overestimation may be due to stolon nodes that are scanned by the software, affecting the total projection surface that is used for calculating diameter (ratio between total projection surface and total length), and instead are excluded when measurements are made with the caliper. The correlation between the number of nodes and difference between diameter values obtained by both methods (measured and scanned) was significant only in stolons (**Figure 3a**); also, variations in the number of nodes explained only a small part of the variation of this difference ( $R^2 = 14\%$ ). The significant correlation found between scanned internode diameter and measured diameter (slopes of 1.01 and 0.98 for stolons and rhizomes, respectively; intercepts of nearly zero) (**Figure 4a** and **4b**) demonstrates that internode diameter can be accurately estimated through the digital image analysis system as long as nodes are removed. Therefore, total stolon length and average diameter of samples composed by numerous stolons or rhizomes can be easily and accurately quantified using the digital image analysis system.

As part of an ongoing experiment, one turf sample (20 x 20 x 15 cm depth) was collected in each plot seasonally from autumn 2013 to summer 2015 and was handled as described in the protocol. The stolon and rhizome length per unit surface area (length density) and weight per unit surface area (weight density) of samples collected in July 2014 are presented in **Figure 5**. Differences in stolon length density were observed between the vegetatively propagated cultivars ("Patriot" and "Tifway") and seeded ones ("La Paloma" and "Yukon"). "Patriot" displayed the highest rhizome length density, followed by "Tifway" and the seeded cultivars. The stolon weight density was different for all cultivars, with "Patriot" showing the highest value followed by "Tifway", "La Paloma" and "Yukon". The vegetatively propagated cultivars also displayed higher rhizome weight densities than the seeded cultivars. The development of stolon and rhizome length per unit surface area (length density) and weight per unit surface area (weight density) of cultivar Patriot throughout the study period are reported in **Figure 6**. Stolon length density displayed an increase from March 2014 to July 2014, and it did not vary from July 2014 to July 2015. Only a few rhizomes were found in samples collected in October 2013 and March 2014. Rhizome length density increased in July 2014, reaching its highest values, but decreased again in October 2014. Stolon weight density slightly increased from March to July 2014; however, a more rapid increase was observed from July to October 2014, with a subsequent decrease in March 2015. Rhizome weight density had a similar trend to rhizome length density, with its highest value in July 2014.

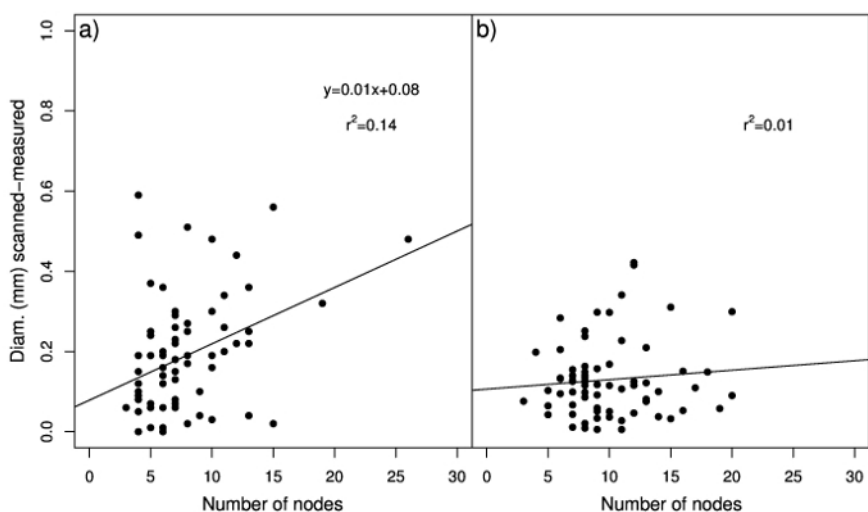
The software includes in the analysis all objects in the scanned image. An example of a digital image analysis layout from WinRHIZO software is presented (**Figure 7**), where lines of different color overlay objects (stolons) of varying diameter to calculate the total length per diameter class. We can observe that the analysis takes into account fragments of roots or leaves. As described in the step 3.9, it is possible to restrict the width and number of diameter classes that are analyzed. The histogram shows the distribution of lengths into selected diameter classes (**Figure 7**). This histogram can be used to assess the minimum diameter classes to be excluded. A visual observation of this graph in the upper part of the screen image highlights that the length has a normal distribution around an average mean diameter class, with the exception of the first two classes that show higher values than those fitting the normal distribution. Even if samples have been carefully cleaned, including these smaller classes, data analysis may affect the results, overestimating length density and underestimating average diameter. Our results show that the length of smaller classes (diameter < 0.2 mm) accounted for 13-32% of the total rhizome length values resulting from the software analysis (**Table 1**). Moreover, average diameter was underestimated from 2-17% (**Table 1**).



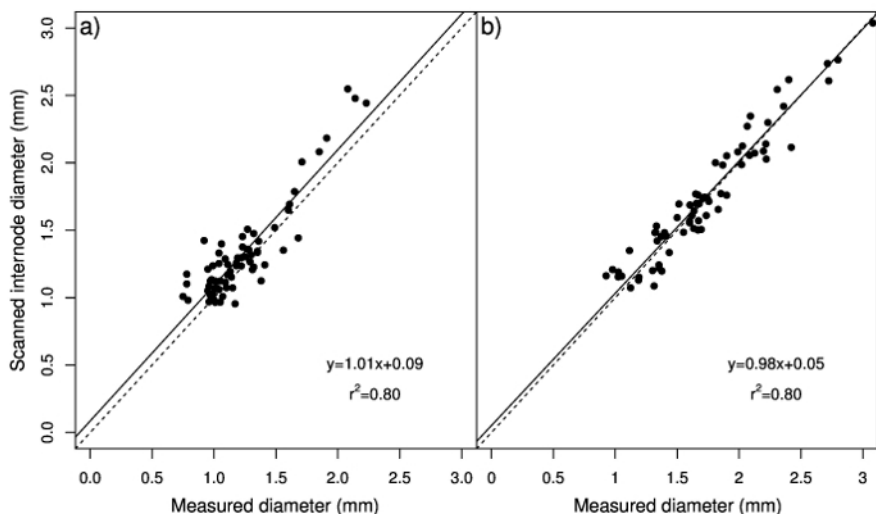
**Figure 1: Regression analysis of length values measured with the ruler against values estimated with the digital image analysis system of bermudagrass stolons<sup>24</sup> (a) and rhizomes (b).** The dashed line represents a 1:1 ratio. Panel A has been modified from Pomaro *et al.*<sup>24</sup>. Please click [here](#) to view a larger version of this figure.



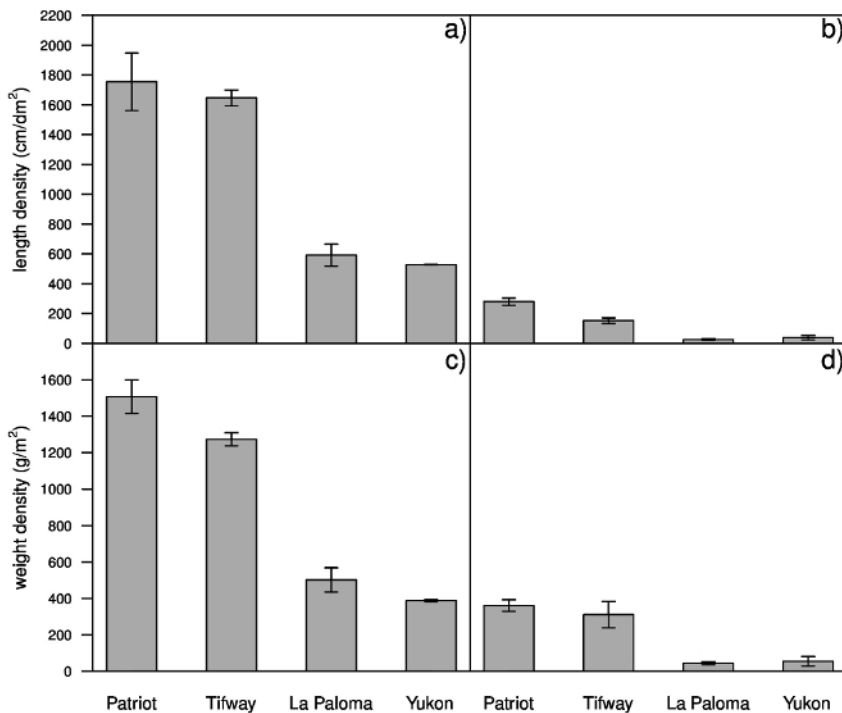
**Figure 2: Regression analysis of diameter values measured with the caliper against values estimated with the digital image analysis system of bermudagrass stolons<sup>24</sup> (a) and rhizomes (b).** The dashed line represents a 1:1 ratio. Panel A has been modified from Pornaro *et al.*<sup>24</sup>. [Please click here to view a larger version of this figure.](#)



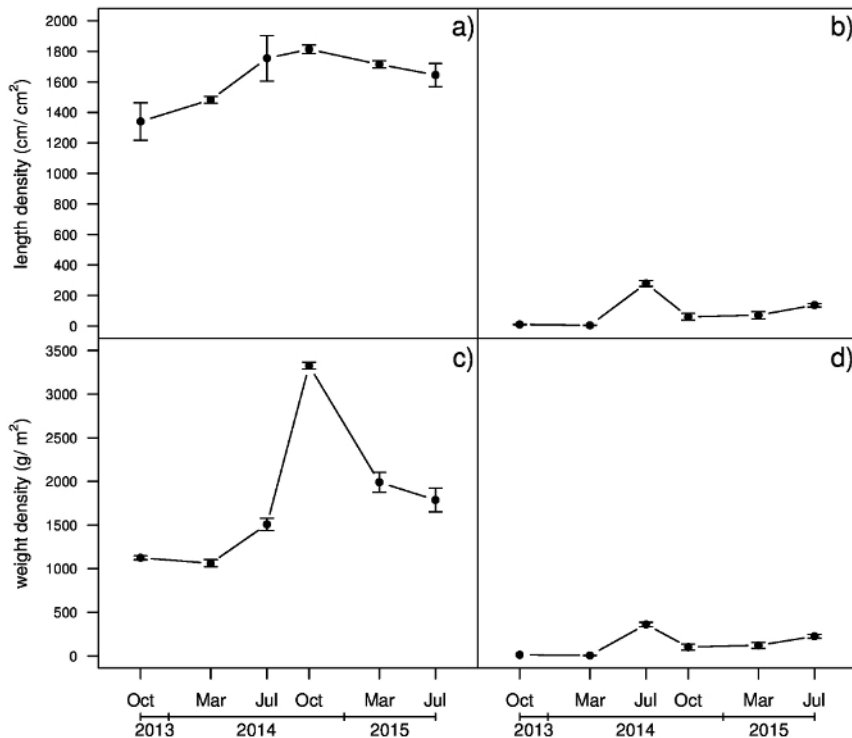
**Figure 3: Regression analysis of number of nodes of bermudagrass stolons<sup>24</sup> (a) and rhizomes (b) against absolute values of difference between diameter estimated with the digital image analysis system and measured with the caliper.** The dashed line represents a 1:1 ratio. Panel A has been modified from Pornaro *et al.*<sup>24</sup>. [Please click here to view a larger version of this figure.](#)



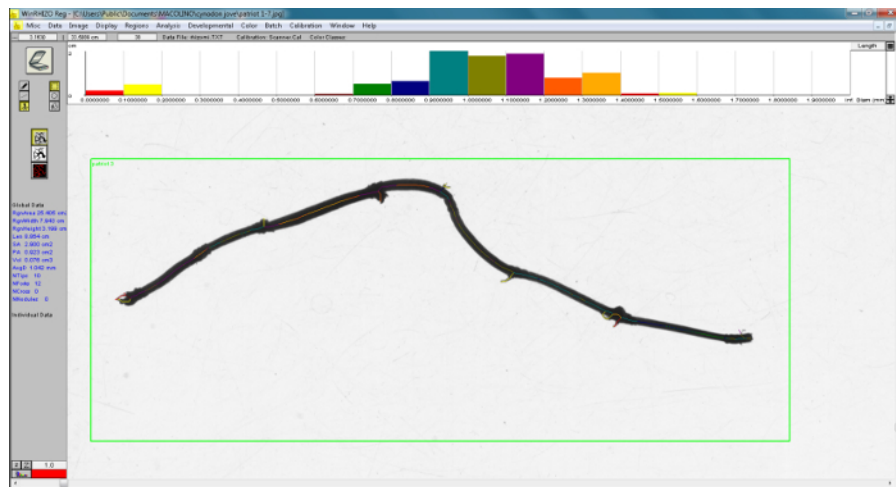
**Figure 4: Regression analysis of diameter values measured with the caliper against values estimated with the digital image analysis system of bermudagrass stolons<sup>24</sup> (a) and rhizomes (b) for internodes only.** The dashed line represents a 1:1 ratio. Panel A has been modified from Pomaro *et al.*<sup>24</sup>. [Please click here to view a larger version of this figure.](#)



**Figure 5: Example results of length and weight density of stolons and rhizomes from a field trial comparing four turfgrass bermudagrass cultivars (Patriot, Tifway, La Paloma, Yukon).** Stolon length density (a), rhizome length density (b), stolon weight density (c), and rhizome weight density (d). Vertical bars represent standard errors of six replicates. [Please click here to view a larger version of this figure.](#)



**Figure 6: Example results of length and weight density of stolons and rhizomes from a field trial showing stolon and rhizome development of Patriot bermudagrass cultivar.** Stolon length density (a), rhizome length density (b), stolon weight density (c), and rhizome weight density (d). Vertical bars represent standard errors of six replicates. [Please click here to view a larger version of this figure.](#)



**Figure 7: Example layout of digital image analysis from WinRHIZO software.** The scanned image in the foreground and the bar charts in the upper part of the screen image show the length distribution in selected diameter classes. The colored lines indicate the image analysis, and each color corresponds to colors of diameter classes reported in the bar charts. [Please click here to view a larger version of this figure.](#)

Cultivar	Block	Rhizome length (cm/dm <sup>2</sup> )			Average diameter (mm)		
		< 0.2 mm	total	ratio <sup>a</sup>	< 0.2 mm	total	ratio <sup>b</sup>
Patriot	1	231	278	16.9	1.637846	1.5994	97.7
Patriot	2	304	349	12.8	1.620667	1.588371	98.0
Patriot	3	304	366	16.8	1.649918	1.621367	98.3
Tifway	1	184	231	20.6	2.149745	1.9951	92.8
Tifway	2	155	193	19.9	1.866253	1.76605	94.6
Tifway	3	119	150	20.9	1.877386	1.75865	93.7
La Paloma	1	17	23	24.4	2.139019	1.8904	88.4
La Paloma	2	26	38	31.6	2.101385	1.7455	83.1
La Paloma	3	34	47	27.5	2.033729	1.7354	85.3
Yukon	1	32	44	28.0	1.700155	1.4945	87.9
Yukon	2	17	25	33.2	1.68339	1.4284	84.9
Yukon	3	67	87	23.6	1.844721	1.6774	90.9
<sup>a</sup> Length of classes ≤0.2 mm/total length							
<sup>b</sup> Total diameter/diameter of classes ≤0.2 mm							

**Table 1: Rhizome length density and rhizome average diameter with and without smaller diameter classes.** Length density with and without including diameter classes smaller than 0.2 mm and their ratios (length of classes ≤ 0.2 mm/total length); and average diameter with and without including diameter classes smaller than 0.2 mm and their ratios (diameter including classes < 0.2/diameter without including diameter classes < 0.2 mm).

## Discussion

The protocol described here was developed and evaluated for the study of turfgrasses. However, it can be used over a range of stoloniferous or rhizomatous species with necessary adjustments according to their morphological characteristics, environmental conditions, and sample cleaning precision.

The average diameter estimated by means of this protocol cannot be compared to the internode diameter measured with a caliper. The digital image analysis includes nodes and internodes in the calculation of average diameter, that is the ratio between total projection surface and total length. As discussed by Pornaro *et al.*<sup>24</sup>, average diameter obtained for bermudagrass stolons with WinRHIZO system overestimated average diameter values measured with the caliper at the internode. Stolon diameter is typically used to describe the diameter of stolon internodes and is a common parameter used for botanical description<sup>18,25</sup>. For this reason, Pornaro *et al.*<sup>24</sup> pointed out that average diameter estimated through WinRHIZO system and the manually measured internode diameter describe two different morphology aspects.

The time required to perform this protocol remains a limiting factor for routine analysis. The most time-consuming phase is the cleaning of the samples (step 2.4). Based on our experience, cleaning one turf sample with a large amount of biomass (*i.e.*, 20 x 20 cm) requires approximately three people working for 2 to 4 hours. As described in the protocol, the cleaning process is necessary for both the digital analysis system and when using the caliper and ruler. When samples consist of a limited number of stolons/rhizomes, the time necessary to collect data with the two methods is similar. However, as sample size is increased, the software-based method does not have a subsequent time increase, as the only limiting factor is the surface area of the scanner. On the contrary, the time necessary to measure organs with the ruler and caliper increases with the number of stolons or rhizomes composing the sample.

The study of stolon and rhizome traits in mature turfgrasses has always been based on the measurement of internode length and diameter and mass dry weight<sup>7,11,26,27</sup>. Due to the large time required to process samples and the decrease in accuracy with the increase of sample size, manual measurements should be limited to a small number of stolons or rhizomes<sup>11,20,25</sup>. As such, they may only be suitable for single-plant experiments. The advantage of an image analysis system over traditional methods is that it that can measure the length of large stolon or rhizome samples and calculate both length density and specific weight (weight-length ratio).

This protocol allows for the measurement of stolon and rhizome length and calculation of length densities in samples with large biomass (for which stolon or rhizome weight is currently the only parameter used for morphology description). Stolon and/or rhizome length may be an important parameter in many studies that cannot be estimated with current techniques. Recent studies on different turf species<sup>9</sup> have demonstrated that stolon weight and length densities are not always correlated, indicating that it may be desirable to measure multiple parameters to adequately assess the stolon and rhizome system. This method should be particularly suitable for cultivar or cultural management practices comparison.

Several steps within the protocol are critical for a successful estimation of length and average diameter of stolons and rhizomes. Because of high variability of plant morphology under different environment conditions, the number of samples (sample size) and ground area dimensions that should be sampled (sample dimension) should be carefully evaluated and be as representative as possible of the population in order to reduce data variability. Moreover, cleaning roots and leaves from the stolons before analysis is meticulous work requiring special attention to avoid overestimations. Lastly, before processing images, it is recommended to carefully select the width of diameter classes and minimum



diameter using software options to exclude everything that is not a stolon or rhizome from the analysis. Each experiment requires the selection of a minimum diameter, as diameter varies with species and environmental conditions, including cultural practices.

## Disclosures

The authors have nothing to disclose.

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